

A Specific *CNOT1* Mutation Results in a Novel Syndrome of Pancreatic Agenesis and Holoprosencephaly through Impaired Pancreatic and Neurological Development

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We report a recurrent *CNOT1* *de novo* missense mutation, GenBank: NM_016284.4; c.1603C>T (p.Arg535Cys), resulting in a syndrome of pancreatic agenesis and abnormal forebrain development in three individuals and a similar phenotype in mice. *CNOT1* is a transcriptional repressor that has been suggested as being critical for maintaining embryonic stem cells in a pluripotent state. These findings suggest that *CNOT1* plays a critical role in pancreatic and neurological development and describe a novel genetic syndrome of pancreatic agenesis and holoprosencephaly.

Discovering genes with mutations causal of pancreatic agenesis is crucial to identifying factors needed for pancreatic development. To date, pathogenic variants in six genes (*PTF1A* [MIM: 615935], *PDX1* [MIM: 260370], *GATA6* [MIM: 600001], *GATA4* [MIM: 600576], *HNF1B* [MIM: 137920], and *RFX6* [MIM: 615710]) have been reported to severely affect pancreatic development and result in pancreatic agenesis.¹ Gene discovery in pancreatic agenesis has shown both similarities and marked differences between pancreatic development in human and mouse. In both species, complete loss of function of *PTF1A*, *PDX1*, or *RFX6* results in pancreatic agenesis. In contrast, while haploinsufficiency of *GATA6* is a common cause of pancreatic agenesis in humans,² in mice *Gata6* knockout does not result in abnormal pancreatic development.³ Knowledge of human pancreatic development is essential to guide progress of beta-cell replacement therapy for people with type 1 diabetes.

We investigated an international cohort of 107 individuals diagnosed with pancreatic agenesis—defined by requiring both endocrine (insulin) and exocrine (pancreatic enzymes) replacement therapy within the first 6 months of life—and identified a mutation in a known gene in 98 of them (Table S1). To identify *de novo* mutations in the remaining nine subjects, exome sequencing was performed for the probands and both their unaffected parents when available (n = 7) (Supplemental Subjects and Methods).

We identified a heterozygous missense mutation in *CNOT1* (MIM: 604917; GenBank: NM_016284.4; c.1603C>T [p.Arg535Cys]) in three individuals with pancreatic agenesis. The variant had arisen *de novo* in two of them and was not present in the DNA sample from the 3rd individual's father (maternal sample was not available for testing) (Figure 1A, Tables S2 and S3). We confirmed these results by Sanger sequencing (Supplemental Subjects and Methods, Figure S1). The p.Arg535Cys variant is absent from dbSNP138, DECIPHER, and GnomAD and affects a residue which is highly conserved across species (up to *C. elegans*) (Figure 1B). All three *in silico* prediction tools used (AlignGVGD, PolyPhen2, and SIFT accessed through AlamutVisual) predicted the variant to have a deleterious effect on protein function (Supplemental Subjects and Methods).

The three individuals who were heterozygous for the *CNOT1* p.Arg535Cys variant had strikingly similar clinical features (see Supplemental Note). In addition to pancreatic agenesis, all three had definite (n = 2) or possible holoprosencephaly (Figure 1A, Table S4), a disorder in which the prosencephalon (forebrain of the embryo) fails to develop into two hemispheres. P01 and P02 (who was previously reported by Hilbrands et al.⁴) both had partial/semi-lobar holoprosencephaly, while P03 has dysmorphic features which could be consistent with holoprosencephaly (prominent central incisors and occiput, highly arched palate, and low-set ears) but brain MRI was declined by his parents

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<https://doi.org/10.1016/j.ajhg.2019.03.018>

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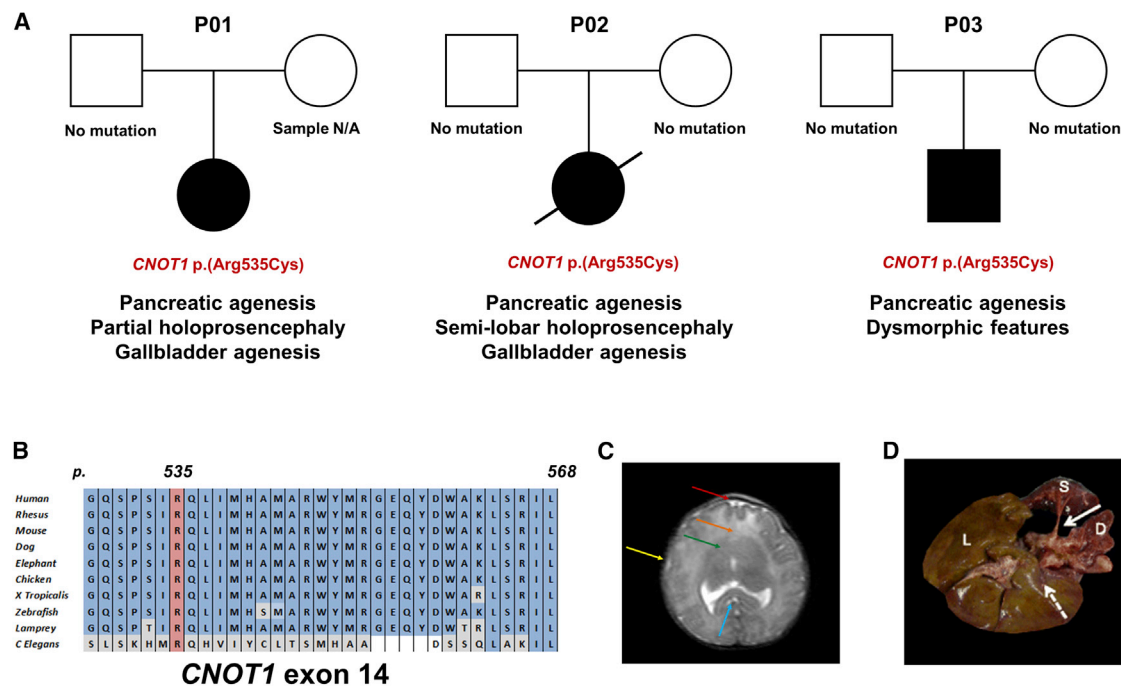


Figure 1. Genetic and Clinical Findings in Individuals with Pancreatic Agenesis

and the diagnosis could not therefore be confirmed. All three individuals had very low birth weight (Z-score < -2), likely due to insulin deficiency in the last trimester of pregnancy, when insulin is the main fetal growth factor. Consistent with insulin deficiency *in utero*, the three case subjects all developed diabetes very early (2/3 diagnosed at 1 day and 1 at 13 weeks). P01 and P02 also had gall-bladder agenesis, a clinical feature frequently associated with pancreatic agenesis.

expected Mendelian ratios and were therefore collected to assess their phenotype ([Supplemental Subjects and Methods](#)). Upon dissection, several gross morphological abnormalities were apparent in homozygotes, notably exencephaly, eye defects (mostly coloboma), and edema ([Figures 2A, 2B, and S2; Table S6](#)).

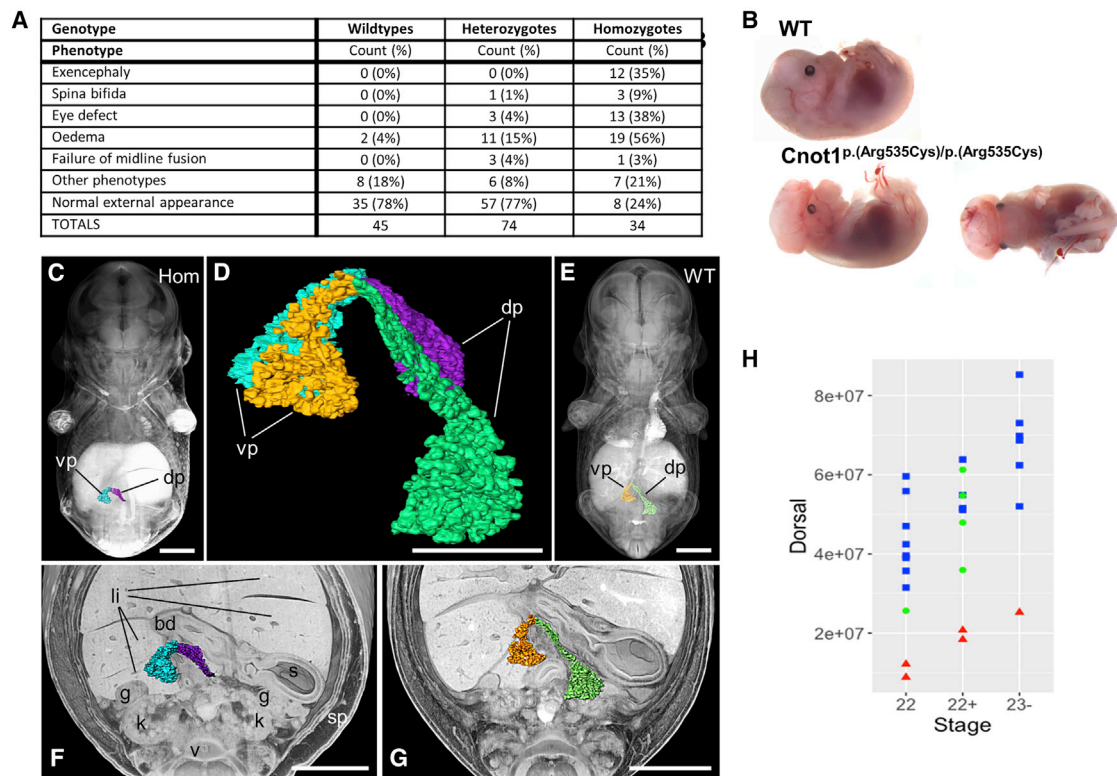


Figure 2. Neurological and Pancreatic Abnormalities in Mouse Embryos Homozygous for the *Cnot1* p.Arg535Cys Mutation

(A) Table listing the gross external phenotypes observed in E14.5 embryos. Numbers do not add to total as many embryos displayed multiple phenotypes. Significance by Fisher's exact test, assuming an additive model. Exencephaly, $p = 3.2 \times 10^{-9}$; spina bifida, $p = 0.027$; eye defect, $p = 5.5 \times 10^{-8}$; edema, $p = 2.6 \times 10^{-7}$; midline defect, ns.

(B) Images showing representative E14.5 embryos: top shows wild-type embryo, bottom shows embryo homozygous for the *CNOT1* p.Arg535Cys mutation with exencephaly and coloboma.

(C and E) Coronal sectioned, semi-transparent 3D volume models of stage-matched E14.5 embryos with superimposed models of the pancreas of homozygous (C) and wild-type (E) embryos.

(D) Overlay of extracted surface models of the pancreas of homozygous (blue, magenta) and wild-type embryos (orange, green).

(F and G) Coronal sectioned solid 3D volume rendered model of the abdomen of the embryos shown in (C) and (E) with superimposed pancreas. dp, dorsal pancreas; vp, ventral pancreas; li, liver lobes; s, stomach; sp, spleen; k, kidney; g, gonad; bd, bile duct.

Scale bars: 1,000 μm in (C), (E)–(G); 500 μm in (D).

(H) Graph showing the volume of the dorsal pancreas of E14.5 embryos in μm^3 . Blue squares show wild types, green circles are heterozygotes, and red triangles are homozygotes. Data analyzed using ANOVA with TukeyHSD posthoc test, effect of genotype $p = 8.85 \times 10^{-8}$; post hoc WT-Hom, $p < 10^{-10}$; Het-Hom, $p = 1.36 \times 10^{-4}$, WT-Het, ns.

disease. Mice required a homozygous mutation in *Cnot1* to display a pancreatic and brain phenotype while a heterozygous *CNOT1* mutation resulted in the phenotype in three individuals in our cohort. This has been described with other pancreatic developmental genes (e.g., *HNF1B*) and supports the hypothesis that the early stages of pancreatic development are not identical in mice and humans.⁶

The *CNOT1* protein has not previously been suggested to have a role in pancreatic development; it is known to act both as scaffold of the CCR4-NOT complex and as an independent factor. As such, it mediates transcriptional repression⁷ and is expressed extremely early during embryonic development (E3.5 in the inner cell mass in mice⁸). *In vitro* studies have proposed that *CNOT1* plays a critical role in maintaining human and mice embryonic stem cells in a pluripotent state by inhibiting primitive endoderm factors.⁸ *CNOT1* expression peaks in undifferentiated

human induced pluripotent (iPS) cells compared to subsequent stages of *in vitro* differentiation toward pancreatic endocrine cells,⁹ supporting its fundamental role in stem cells.

The increased expression of *Shh* in pancreatic tissue extracted from *Cnot1*^{p.Arg535Cys}/p.Arg535Cys embryos would be consistent with a model in which the *CNOT1* p.Arg535Cys mutation results in embryonic stem cells being maintained in an undifferentiated state through SHH-mediated inhibition of differentiation. SHH is a key developmental factor that is known to be crucial for pancreatic and brain development. Heterozygous loss-of-function mutations in *SHH* cause holoprosencephaly (MIM: 142945) and studies in both mouse and human embryos have shown that *SHH* expression needs to be repressed in the dorsal foregut endoderm for successful differentiation toward dorsal pancreas.^{6,10} A recent study has suggested that the transcription factors *Gata4* and *Gata6* (mutations in which are a

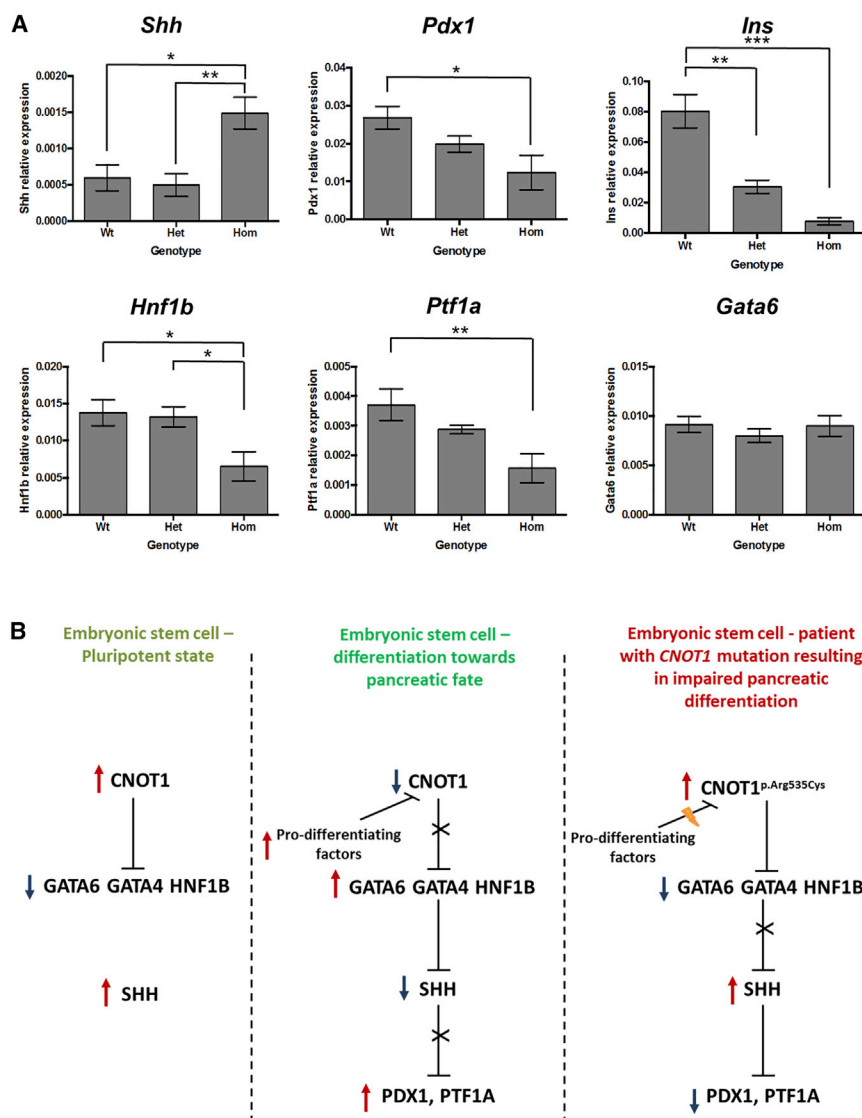


Figure 3. Expression Data and Possible Mechanism Involving CNOT1 in Pancreatic Development

(A) Graphs showing relative expression of genes in the pancreas of E14.5 embryos. Bars show mean \pm SE. Data analyzed using ANOVA with TukeyHSD posthoc test. Results of posthoc tests shown on graphs, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *Shh*, effect of genotype $p = 0.0107$; *Pdx1*, effect of genotype $p = 0.0189$; *Ins*, effect of genotype $p = 7.03 \times 10^{-6}$; *Hnf1b*, effect of genotype $p = 0.00781$; *Ptf1a*, effect of genotype $p = 0.00781$; *Gata6*, effect of genotype $p = \text{ns}$. $n = 4\text{--}12$ animals per genotype. (B) Schematic representation of the proposed role for CNOT1 in pancreatic development.

sion during brain development. This would be consistent with previous reports of *Shh* ectopic expression impairing midline development.¹² Another possibility is that the effect of the *CNOT1* mutation on SHH signaling differs between the brain and the pancreas, resulting in a reduced expression in the developing brain and increased expression during pancreatic development. Further experiments, ideally on younger embryos and human iPS cells, are needed in order to elucidate the mechanism by which the *CNOT1* p.Arg535Cys mutation results in impaired pancreatic and neurological development.

Our study identifies a spontaneous *CNOT1* p.Arg535Cys mutation as the genetic cause of a rare syndrome of

cause of pancreatic agenesis) regulate pancreatic endoderm identity by directly inhibiting *Shh* in mice.¹¹ It is therefore possible that the p.Arg535Cys variant results in *CNOT1* maintaining its inhibition activity on the GATA and other early differentiation factors and, as a consequence, SHH expression is not repressed (Figure 3B). Increased expression of *Shh* and decreased expression of *Pdx1*, *Ins*, *Hnf1b*, and *Ptf1a* detected in RNA extracted from pancreatic tissue in the E14.5 *Cnot1*^{p.(Arg535Cys)/p.(Arg535Cys)} embryos would support this hypothesis. However, *Gata4* expression could not be assessed as the assay specificity was too low and *Gata6* expression was not found to be reduced. It is possible that *Gata6* activity is actually inhibited earlier during development and then re-activated by a different pathway (*Gata6* is needed for development of most endodermal-derived organs and heart) or could be inhibited by a different mechanism that does not result in reduced expression. The *CNOT1* p.Arg535Cys mutation also affects neurological development in both our case subjects and mouse embryos. It is possible that this mutation results in ectopic SHH expres-

pancreatic agenesis and holoprosencephaly, highlighting a previously unsuspected role of *CNOT1* as a key factor in both pancreatic and neurological development. This is the 7th gene causative of pancreatic agenesis described so far and the first pancreatic agenesis gene that is thought to be important for maintaining stem cells' pluripotency. These findings suggest a new mechanism by which impairment of the very early stages of development result in pancreatic agenesis and abnormal brain development.

Supplemental Data

Supplemental Data can be found online at <https://doi.org/10.1016/j.ajhg.2019.03.018>.

Acknowledgments

The authors thank the families for participating in this study. We are grateful to Anna-Marie Johnson and Benjamin Bunce for their technical assistance and to Dr. Jane Ferguson and Dr. Joerg Detlev Moritz for assistance with the MRI images. We are grateful to

Emma Siragher and Monika Dabrowska for assistance with embryo dissection, Antonella Galli for advice on embryo work, members of the DMDD consortium at the Francis Crick Institute for imaging the embryos, Julia Rose for assistance with HREM image analysis, members of the Research Support Facility and Mouse Pipelines at the Wellcome Sanger Institute for mouse management and husbandry, Rachel Moore and Eleanor Wheeler for help with the mouse statistical analysis, Bill Bottomley for help with mouse generation, and Elizabeth Wynn for help with genotyping. We would also like to thank Prof. Lori Sussell for useful discussion on the SHH pathway.

I.B. is funded by Wellcome (WT206194). A.T.H. and S.E. are the recipients of a Wellcome Trust Senior Investigator award (grant WT098395/Z/12/Z) and A.T.H. is employed as a core member of staff within the NIHR funded Exeter Clinical Research Facility and is an NIHR senior investigator. E.D.F. was a Naomi Berrie Fellow in Diabetes Research (grant 50565) during the study. S.E.F. has a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (grant number 105636/Z/14/Z). C.C.W. holds a Wellcome Trust Intermediate Clinical Fellowship (grant number 105914/Z/14/Z). H.H. is funded by the Research Foundation-Flanders (FWO), the VUB Research Council, and Stichting Diabetes Onderzoek Nederland.

Declaration of Interests

The authors declare no competing interests.

Received: December 3, 2018

Accepted: March 18, 2019

Published: April 18, 2019

Web Resources

DECIPHER, <https://decipher.sanger.ac.uk/>

DMDD, <https://dmdd.org.uk>

GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>

gnomAD Browser, <https://gnomad.broadinstitute.org/>

OMIM, <http://www.omim.org/>

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